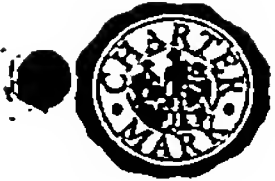


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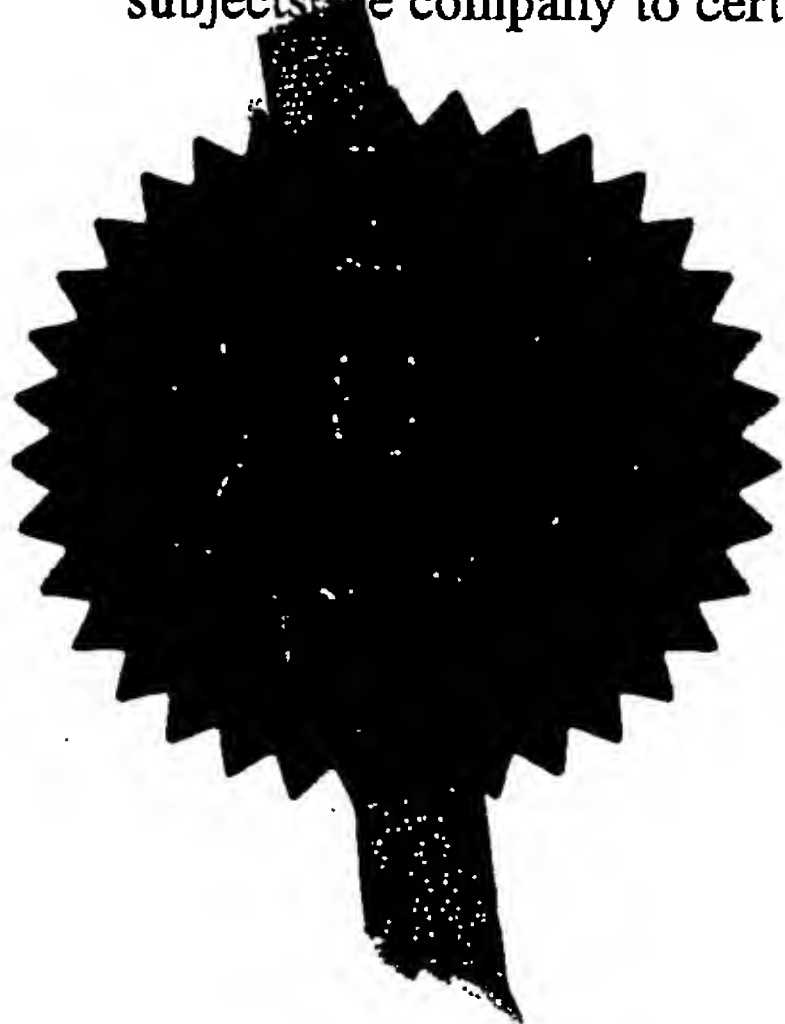
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1. Your reference

P33120-/LMC/MCM

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28 DEC 2002

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

Fusion Antibodies Limited
PO Box 374
Belfast
BT1 2WD

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

08295982001

4. Title of the invention

"Purification Means"

5. Name of your agent (*if you have one*)

Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent (*including the postcode*)

Scotland House
165-169 Scotland Street
Glasgow
G5 8PL

Patents ADP number (*if you know it*)

1198013

1198013

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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (*Answer 'Yes' if:*

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 - b) there is an inventor who is not named as an applicant, or
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Description

33

Claim(s)

4

Abstract

-

Drawing(s)

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Priority documents

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

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I/We request the grant of a patent on the basis of this application.

Signature

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Date

27 December 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Malcolm C Main

0141 307 8400

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Patents Form 1/77

1 Purification Means

2

3 The present invention relates to purification means,
4 in particular to means suitable for use in
5 purification of soluble proteins.

6

7 Introduction

8

9 The recombinant production of protein in bacteria,
10 yeast, insect and mammalian cell lines has become a
11 cornerstone of biological research and the
12 biotechnology industry. Classical biochemical and
13 chromatographical purification techniques usually
14 produce inadequate amounts of a target protein to
15 study its roles or actions. Even if enough of the
16 protein can be purified, it usually involves
17 cumbersome amounts of starting material or tissue
18 and many processing steps are taken before
19 reasonable purification can be achieved.

20

21 Recombinant expression of the target protein
22 bypasses a lot of these problems. By introducing

1 the target protein's gene template to a cell line or
2 bacterial culture, induced overexpression can result
3 in significant levels of that protein being
4 produced. Large amounts of protein make the
5 purification a lot simpler, but the addition or
6 fusion of purification domains or tags allows for a
7 relatively simple one-step purification using
8 affinity chromatography resins. However,
9 occasionally, due to the varying nature of proteins,
10 the production of soluble protein has remained
11 elusive with known tags unable to purify many
12 proteins. In some cases, production of protein can
13 be a problem due to differences in the machinery of
14 bacterial cells. There is therefore a need for a
15 more versatile tag than is available currently on
16 the market. The provision of such a versatile tag
17 enabling, for example, improved ability to quickly
18 produce and screen soluble protein in bacteria such
19 as *E.coli* would represent a major step forward in
20 protein biochemistry.

21

22 Summary of the Invention

23

24 The present inventors have developed a novel
25 purification tag based on the gene product of a
26 sortase gene, in particular the *srtA* gene of
27 *Staphylococcus aureus*. This tag, known as SNUT
28 [Solubility eNhancing Unique Tag] has been found to
29 have exceptional activity, enabling the efficient
30 purification of soluble domains of a number of
31 proteins hitherto not able to be isolated
32 efficiently using conventional purification tags.

1
2 Throughout this specification, reference to a SNUT
3 Tag should be understood to mean a tag derived from
4 a sortase gene product.

5
6 In a first aspect of the invention, there is
7 provided a purification tag comprising a sortase,
8 e.g srtA, gene product.

9
10 In preferred embodiments, the sortase gene product
11 is a gene product of the srtA gene of *Staphylococcus*
12 *aureus*.

13
14 Also provided is the use of a sortase, e.g srtA,
15 gene product as a purification tag.

16
17 Furthermore, according to a third aspect of the
18 invention, there is provided an expression construct
19 for the production of recombinant polypeptides,
20 which construct comprises an expression cassette
21 consisting of the following elements that are
22 operably linked: a) a promoter; b) the coding region
23 of a DNA encoding a sortase, eg srtA gene product as
24 a purification tag sequence; c) a cloning site for
25 receiving the coding region for the recombinant
26 polypeptide to be produced; and d) transcription
27 termination signals.

28
29 According to a fourth aspect of the invention, there
30 is provided a method for producing a polypeptide,
31 comprising: a) preparing an expression vector for
32 the polypeptide to be produced by cloning the coding

1 sequence for the polypeptide into the cloning site
2 of an expression construct according to the third
3 aspect of the invention; b) transforming a suitable
4 host cell with the expression construct thus
5 obtained; and c) culturing the host cell under
6 conditions allowing expression of a fusion
7 polypeptide consisting of the amino acid sequence of
8 the purification tag with the amino acid sequence of
9 the polypeptide to be expressed covalently linked
10 thereto; and, optionally, d) isolating the fusion
11 polypeptide from the host cell or the culture medium
12 by means of binding the fusion polypeptide present
13 therein through the amino acid sequence of the
14 purification tag.

15

16 The expression construct, herein referred to as
17 pSNUT, may be made by modification of any suitable
18 vector to include the coding region of a DNA
19 encoding a sortase. In preferred embodiments, the
20 expression construct is based on the pQE30 plasmid.

21

22 A sample of pSNUT was deposited with the National
23 Collections of Industrial and Marine Bacteria Ltd.
24 (NCIMB), 23 St Machar Drive, Aberdeen, Scotland AB24
25 3RY on 23 December 2002 under accession no NCIMB
26 41153.

27

28 In a fifth aspect, there is provided a fusion
29 polypeptide obtained by the method of the fourth
30 aspect of the invention.

31

1 In preferred embodiments, the sortase, e.g.
2 srtA, gene product (SNUT) is encoded by the
3 nucleotide sequence shown in Figure 4 or a variant
4 or fragment thereof. Preferably, the srtA gene
5 product comprises amino acids 26 to 171 of the SrtA
6 sequence shown in Figure 4 or a variant or fragment
7 thereof.

8
9 Variants and fragments of and for use in the
10 invention preferably retain the functional
11 capability of the polypeptide i.e. ability to be
12 used as a purification tag. Such variants and
13 fragments which retain the function of the natural
14 polypeptides, can be prepared according to methods
15 for altering polypeptide sequence known to one of
16 ordinary skill in the art such as are found in
17 references which compile such methods, e.g.
18 Molecular Cloning: A Laboratory Manual, J. Sambrook,
19 et al., eds., Second Edition, Cold Spring Harbor
20 Laboratory Press, Cold Spring Harbor, New York,
21 1989, or Current Protocols in Molecular Biology, F.
22 M. Ausubel, et al., eds., John Wiley & Sons, Inc.,
23 New York.

24
25 A variant nucleic acid molecule shares homology
26 with, or is identical to, all or part of the coding
27 sequence discussed above. Generally, variants may
28 encode, or be used to isolate or amplify nucleic
29 acids which encode, polypeptides which are capable
30 of ability to be used as a purification tag.

31

1 Variants of the present invention can be artificial
2 nucleic acids (i. e. containing sequences which have
3 not originated naturally) which can be prepared by
4 the skilled person in the light of the present
5 disclosure. Alternatively they may be novel,
6 naturally occurring, nucleic acids, which may be
7 isolatable using the sequences of the present
8 invention. Thus a variant may be a distinctive part
9 or fragment (however produced) corresponding to a
10 portion of the sequence provided in Figure 4. The
11 fragments may encode particular functional parts of
12 the polypeptide.

13
14 The fragments may have utility in probing for, or
15 amplifying, the sequence provided or closely related
16 ones.

17
18 Sequence variants which occur naturally may include
19 alleles or other homologues (which may include
20 polymorphisms or mutations at one or more bases).
21 Artificial variants (derivatives) may be prepared by
22 those skilled in the art, for instance by site
23 directed or random mutagenesis, or by direct
24 synthesis. Preferably the variant nucleic acid is
25 generated either directly or indirectly (e. g. via
26 one or amplification or replication steps) from an
27 original nucleic acid having all or part of the
28 sequences of Figure 4. Preferably it encodes a
29 polypeptide which can be used as a purification
30 tag.

31

1 The term 'variant' nucleic acid as used herein
2 encompasses all of these possibilities. When used in
3 the context of polypeptides or proteins it indicates
4 the encoded expression product of the variant
5 nucleic acid.

6
7 Homology (i. e. similarity or identity) may be as
8 defined using sequence comparisons are made using
9 FASTA and FASTP (see Pearson & Lipman, 1988. Methods
10 in Enzymology 183 : 6398). Parameters are preferably
11 set, using the default matrix, as follows :

12 Gapopen (penalty for the first residue in a gap) :-

13 12 for proteins/-16 for DNA

14 Gapext (penalty for additional residues in a gap) :-

15 2 for proteins/-4 for DNA

16 KTUP word length : 2 for proteins/6 for DNA.

17 Homology may be at the nucleotide sequence and/or
18 encoded amino acid sequence level. Preferably, the
19 nucleic acid and/or amino acid sequence shares at
20 least about 60%, or 70%, or 80% homology, most
21 preferably at least about 90%, 95%, 96%, 97%, 98% or
22 99% homology with the sequence shown in Figure 4.

23
24 Thus a variant polypeptide in accordance with the
25 present invention may include within the sequence
26 shown in Figure 4, a single amino acid or 2, 3, 4,
27 5, 6, 7, 8, or 9 changes, about 10, 15, 20, 30, 40
28 or 50 changes. In addition to one or more changes
29 within the amino acid sequence shown, a variant
30 polypeptide may include additional amino acids at
31 the C terminus. and/or N-terminus.

32

1 Naturally, regarding nucleic acid variants, changes
2 to the nucleic acid which make no difference to the
3 encoded polypeptide (i.e. 'degeneratively
4 equivalent') are included within the scope of the
5 present invention.

6
7 Preferred variants include one or more of the
8 following changes (using the annotation of AF162687):
9 nucleotide 604 AAG causing an amino acid mutation of
10 KAR; nucleotide 647 AAG, codon remains K, therefore
11 a silent mutation; nucleotide 966 GAA causing an
12 amino acid mutation of GAQ.

13
14 Changes to a sequence, to produce a derivative, may
15 be by one or more of addition, insertion, deletion
16 or substitution of one or more nucleotides in the
17 nucleic acid, leading to the addition, insertion,
18 deletion or substitution of one or more amino acids
19 in the encoded polypeptide. Changes may be by way of
20 conservative variation, i. e. substitution of one
21 hydrophobic residue such as isoleucine, valine,
22 leucine or methionine for another, or the
23 substitution of one polar residue for another, such
24 as arginine for lysine, glutamic for aspartic acid,
25 or glutamine for asparagine. As is well known to
26 those skilled in the art, altering the primary
27 structure of a polypeptide by a conservative
28 substitution may not significantly alter the
29 activity of that peptide because the side-chain of
30 the amino acid which is inserted into the sequence
31 may be able to form similar bonds and contacts as
32 the side chain of the amino acid which has been

1 substituted out. This is so even when the
2 substitution is in a region which is critical in
3 determining the peptides conformation.

4
5 Also included are variants having non-conservative
6 substitutions. As is well known to those skilled in
7 the art, substitutions to regions of a peptide which
8 are not critical in determining its conformation may
9 not greatly affect its activity because they do not
10 greatly alter the peptide's three dimensional
11 structure.

12
13 In regions which are critical in determining the
14 peptides conformation or activity such changes may
15 confer advantageous properties on the polypeptide.
16 Indeed, changes such as those described above may
17 confer slightly advantageous properties on the
18 peptide e. g. altered stability or specificity.

19
20 SNUT tags and vectors may be used in methods of
21 purifying a soluble domain of a peptide.
22 Accordingly in a further aspect of the invention,
23 there is provided a method of producing a soluble
24 bioactive domain of a protein, the method
25 comprising the steps of cloning DNA encoding at
26 least one candidate soluble domain into at least one
27 expression vector, transfecting or transforming a
28 host cell with said vector, expressing said DNA in
29 said host cell, wherein said vector encodes a
30 sortase gene product.

31

1 The sortase gene product is preferably in the form
2 of a fusion protein.

3

4 The method may comprise the steps of analysis of DNA
5 coding for the protein of interest to identify
6 antigenic soluble domains, designing oligonucleotide
7 primers to amplify DNA encoding the domain,
8 amplifying DNA, cloning the DNA, optionally
9 screening clones for correct orientation of DNA,
10 expressing DNA in expression strains, analysing
11 expression products for solubility, analysing
12 products and production of soluble bioactive protein
13 domain.

14

15 The method optionally comprises the step of
16 producing a soluble bioactive protein domain of said
17 protein of interest.

18

19 The invention is exemplified with reference to the
20 following non limiting description and the
21 accompanying figures in which

22

23 Figure 1 shows selected domains for amplification
24 from *in silico* analysis. Representation of a
25 candidate protein for the expression platform, in
26 this case Jak1 (human). Four fragments have been
27 chosen by analysis as depicted.

28

29 Figure 2 shows denaturing dot-blot analysis of
30 expression clones of fragments of MAR1 in pQE30.

31

1 Figure 3 shows a ribbon Diagram of *Staphylococcus*
2 *aureus* sortase. Ribbon diagram of the putative
3 structure of *S. aureus* SrtA protein (minus its N-
4 terminal membrane anchor). SNUT represents the
5 portion of this structure between the two yellow
6 arrows as shown. The yellow ball signifies a Ca^{2+}
7 ion, essential for the biological activity of this
8 protein. This diagram is taken from Hlangovan et
9 al., 2001, PNAS 98 (11) 6056
10 (doi:10.1073/pnas.101064198)

11
12 Figure 4 shows the Nucleotide Sequence and amino
13 acid sequence of SNUT fragment

14
15 (a) This is the determined sequence of SNUT. The
16 fragment was cloned into pQE30 using the *Bam*HI site
17 of this vector. When in the wanted orientation,
18 insertion results in the inactivation of the
19 upstream cloning site, therefore allowing any
20 subsequent cloning of target inserts with the
21 downstream *Bam*HI site (see (b) for restriction map
22 of sequence).

23
24 Figure 5 illustrates qualitative purification
25 results using the SNUT fusion tag. (a) shows the
26 elution profile on SDS-PAGE of SNUT-Jak1 using AKTA
27 Prime native histag purification. Successful
28 elution of SNUT-Jak1 construct is signified by the
29 white arrow. (b) shows the elution profile on SDS-
30 PAGE of SNUT-MAR1 using AKTA Prime native histag
31 purification. Successful elution is shown by the
32 arrow. (c) shows the same gel stained in (b)

1 western blotted and detected using poly-histidine-
2 HRP antibody. This is confirmation that the eluted
3 species in (b) is actually SNUT-MAR1, of expected
4 molecular weight.

5
6 Template analysis and primer design

7
8 Analysis of the DNA coding for a protein of interest
9 may be performed using software packages such as
10 Vector NTI (Informax, USA) and
11 BLASTP(<http://www.ncbi.nlm.nih.gov/BLAST/>), p-fam (www.sanger.ac.uk/pfam) and TM pred
12 (www.hgmp.mrc.ac.uk) which may be used to identify
13 complete domains within the protein that
14 significantly increase the likelihood of
15 antigenicity and/or solubility when expressed as a
16 subunit of the original protein coding sequence.
17

18
19 In order to increase the possibility of identifying
20 a soluble domain, preferably multiple sub-domains,
21 more preferably at least three sub-domains, for
22 example 3 to 9 sub-domains may be identified for
23 processing.

24
25 Oligonucleotide primers to amplify the selected sub-
26 domains may be designed with the help of
27 commercially available software packages such as the
28 internet software package Primer3 ([http://www-
genome.wi.mit.edu/genome_software/other/primer3.html](http://www-genome.wi.mit.edu/genome_software/other/primer3.html))
29 (Whitehead Institute for Biomedical Research),
30 Vector NTI (www.informaxinc.com) and DNASIS (Hitachi
31 Software Engineering Company (www.oligo.net)).
32

1
2 Typically primers for use in a method of the
3 invention are in the range 10-50 base pairs in
4 length, preferably 15 to 30, for example 20 base
5 pairs in length, with annealing temperatures in the
6 range 45-72°C, more conveniently 55-60°C. Primers
7 may be synthesised using standard techniques or may
8 be sourced from commercial suppliers such as
9 Invitrogen Life Technologies (Scotland) or MWG-
10 Biotech AG (Germany).

11
12 **PCR of Insert**

13
14 The desired inserts which encode the selected sub-
15 domains are amplified using the primers designed
16 specifically for that target gene using standard PCR
17 techniques. The template DNA for amplification can
18 be in the form of plasmid DNA, cDNA or genomic DNA,
19 depending on whatever is appropriate or indeed
20 available. Any suitable DNA polymerase may be used,
21 for example, Platinum Taq, Pfu (www.stratagene.com)
22 or Pfx (www.invitrogen.com). . Any suitable PCR
23 system may be used, for example, the Expand High
24 Fidelity PCR system (Roche, Basel, Switzerland).

25
26 Several different thermocycler conditions may be
27 used with each set of primers. This increases the
28 chance of the PCR working without having to
29 individually optimise each new primer set. Typically
30 the following three programs may be used in the
31 method:

32

- 1 1. A standard PCR programme using the recommended
2 annealing temperature provided with the
3 primers.
- 4 2. A standard PCR programme using 50°C as the
5 temperature for annealing.
- 6 3. A touchdown PCR programme, where the annealing
7 temperature starts at a high temperature e.g
8 65°C for 10 cycles and then gradually decreases
9 the annealing temperature to 50°C over the
10 subsequent e.g 15 cycles.

11
12 Buffer conditions may be adjusted as required, for
13 example with respect to magnesium ion concentration
14 or addition of DMSO for the amplification of
15 difficult templates. Further details of a suitable
16 purification method which may be used with the
17 vector or tag of the invention can be found in our
18 co-pending PCT application, filed on the same day as
19 this application and claiming priority from GB
20 0131026.7.

21
22 The PCR products may be visualised using standard
23 techniques, for example on a 1.5% agarose gel
24 stained with Ethidium Bromide and the bands are cut
25 out of the gel and purified using Mini elute gel
26 extraction Kit (Qiagen, Crawley, England).

27 28 Expression Vectors

29
30 Amplified DNA inserts may be cloned into expression
31 vectors using techniques dictated by the multiple
32 cloning sites of the vector in question. Such

1 techniques are readily available to the skilled
2 person.

3
4 Any suitable expression system can be used in the
5 invention. Preferably, the expression system is
6 prokaryotic. Suitable vectors for use in the method
7 of the invention include any vector which can encode
8 SNUT. [Solubility eNhancing Unique Tag], for example
9 pSNUT. This tag is based on the sequence of a trans-
10 peptidase found on the surface of gram-positive
11 bacteria. This protein is highly soluble, and
12 expressed at very high levels.

13
14 The inventors have found that SNUT is an ideal
15 fusion tag for conferring solubility and expression
16 levels to target protein fragments. SNUT may be
17 cloned into any suitable vector. For the purposes of
18 the examples shown in this application, the sequence
19 incorporating the SNUT fragment is cloned into pQE30
20 (Qiagen, Valencia, CA) in a manner allowing full use
21 of the multiple cloning site (MCS) of this vector
22 for downstream gene insertions.

23
24 Development of pSNUT

25
26 The inventors found that a tag based on the *srtA*
27 gene product from *Staphylococcus aureus* is highly
28 soluble, reacts well to purification schemes and
29 expresses particularly well. It was hypothesised
30 that the incorporation of a portion or domain of
31 this protein could represent a useful fusion tag in
32 the present method, and indeed the expression of any

1 poorly soluble protein in *E. coli*. Using NMR
2 studies, the 3D structure of this protein has been
3 predicted and is shown in Figure 3. We hypothesised
4 that by taking a portion of this structure, we could
5 make a manipulateable protein tag, but not disturb
6 its tertiary structure enough to reduce its highly
7 favourable characteristics listed above. The region
8 of this protein used as a solubility-enhancing tag
9 is depicted by two arrows.

10

11 The SNUT tag was cloned into pQE30. However, it may
12 be cloned into any suitable expression vector.

13 Positive clones may be identified by denaturing dot
14 blots, SDS-PAGE and Western blotting. Final
15 confirmation of these clones was provided by DNA
16 sequencing, and the sequence of the multiple cloning
17 region of the resultant vector is shown in Figure 4.

18

19 Variances in the sequence of the SNUT domain were
20 observed from the sequence for SrtA that has been
21 logged in Genbank (AF162687). The variances are
22 (using the annotation of AF162687) nucleotide 604
23 AΔG causing an amino acid mutation of KΔR;
24 nucleotide 647 AΔG, codon remains K, therefore a
25 silent mutation; nucleotide 966 GΔA causing an amino
26 acid mutation of GΔQ.

27

28 Preliminary trials and native purification showed
29 that the SNUT fragment was very soluble and its
30 characteristics were in no way diminished by
31 truncation, thus showing that SNUT could represent a
32 useful tag domain (data not shown). As described in

1 the Examples, to fully test the abilities of SNUT,
2 we then chose two proteins were soluble protein
3 production had proved impossible using conventional
4 methods and using the other expression systems of
5 the method of the present invention. Surprisingly,
6 we found that, using pSNUT in the method of the
7 invention, these proteins could be produced in
8 soluble form.

9

10 Clone Propagation

11

12 Target insert/expression vector ligations may be
13 propagated using standard transformation techniques
14 including the use of chemically competent cells or
15 electro-competent cells. The choice of the host
16 cell and strain for transformation is dependent on
17 the characteristics of the expression vectors being
18 utilised.

19

20 Bacterial cells, for example, *Escherichia coli*, are
21 the preferred host cells. However, any suitable
22 host cell may be used. In preferred embodiments, the
23 host cells are *Escherichia coli*.

24

25 The vectors may be used to each transfect or
26 transform a plurality of different host cell
27 strains. The set of host cell strains for
28 individual vector may be the same or different from
29 the set used with other vectors.

30

31 In a particularly preferred embodiment of the
32 invention, each vector may be transformed into three

1 *E. coli* strains (for example, selected from
2 Rosetta(DE3)pLacI, Tuner(DE3)pLacI, Origami BL21
3 (DE3)pLacI and TOP10F, Qiagen).

4
5 Where the vectors are pQE based vectors, TOP10F'
6 cells are preferred for the propagation and
7 expression trials of such vectors. The present
8 inventors have identified this strain as a more
9 superior strain for these vectors than either of the
10 recommended strains by the supplier (M15 and
11 SG13009), in terms of ease of use and culture
12 maintenance (only one antibiotic required as to two
13 with M15 or SG13009 (www.qiagen.com)). Other F'
14 strains such as XL1 Blue can be used, but are
15 inferior to the TOP10F' strain, due to lack of
16 expression regulation (results not shown). The use
17 of TOP10F' (Invitrogen) for the propagation and/or
18 expression pQE based vectors forms an independent
19 aspect of the present invention. Other F' strains
20 such as XL1 Blue may also be used, but are inferior
21 to the TOP10F'.

22
23 After transformation, cells may be plated out onto
24 selection plates and propagated for the development
25 of single colonies using standard conditions.

26

27 Propagation of Cells

1
2 The colonies may be used to inoculate duplicate
3 wells in a 96 well plate.
4

5 Typically, each well may contain 200 μ l of LB broth
6 with the appropriate antibiotics. Each plate may be
7 dedicated to one strain of *E. coli* or other host
8 cell which alleviates the problems of different
9 growth rates. The necessary controls are also
10 included on each plate. The plates are then grown
11 up, preferably at 37°C or any other temperature as
12 appropriate to the particular host cell and vector,
13 with shaking, until log phase is reached. This is
14 the primary plate.
15

16 From the primary plate a secondary plate is seeded
17 and then grown. Typically, the secondary plate is
18 be seeded using 'hedgehog' replicators and then
19 grown up to, for example, log phase, chilled to 16°C
20 for 1 hour. Determination of positive clones from
21 these plates may be undertaken using functional
22 studies. Routinely, 6-48 clones for each insert-
23 vector ligation are taken and propagated in culture
24 micro-titre plates containing up to 500 μ l of media.
25 According to the conditions and reagents required,
26 protein production is then induced, and cultures
27 propagated further. Most vectors are under the
28 control of a promoter such as T7, T7lac or T5, and
29 can be easily induced with IPTG during log phase
30 growth. Typically, cultures are propagated in a
31 peptone-based media such as LB or 2YT supplemented
32 with the relevant antibiotic selection marker.

1 These cultures are grown at temperatures ranging
2 from 4-40 °C, but more frequently in the range of
3 20-37 °C depending on the nature of the expressed
4 protein, with or without shaking and induced when
5 appropriate with the inducing agent (usually log or
6 early stationary phase). After induction, growth
7 propagation can be continued for 1-16 hours for a
8 detectable amount of protein to be produced.

9
10 The primary plate is preferably stored at 4°C until
11 the process is complete.

12
13 Colony Screening for Inserts in Correct Orientation
14 The method of the invention may include the step of
15 testing transformants for correct orientation of the
16 inserts. Identification of positive clones can be
17 achieved through a variety of methods, including
18 standard techniques such as digestion analysis of
19 plasmid DNA; colony PCR and DNA sequencing.
20 Alternatively, dot-blotting may be used for the
21 identification of positive clones for example, using
22 a BioDot apparatus (BioRad) containing
23 nitrocellulose membrane (0.45µm pore size) in
24 accordance with the manufacturers' instructions,
25 prior to final confirmation by DNA sequencing.

26
27 The use of this dot blotting method in the platform
28 represents a rapid, reproducible and robust
29 detection method. This particular method is useful
30 for the rapid detection or presence of recombinant
31 protein and allows for a determination of all clones
32 irrespective of solubility and conformation. This

1 may be important at this stage, because
2 conformational structures can inhibit the detection
3 of tag domains if they are not presented properly on
4 the surface of the protein. This can occur as
5 easily with both soluble and insoluble protein.

6
7 As described above, standard colony PCR techniques
8 may be used. For example, transformants may be
9 selected, either manually or using automation such
10 as the Cambridge BioRobotics BioPick instrument, and
11 screened using directional PCR using a primer that
12 encodes for a sequence on the vector such as S Tag
13 or GATA sequence, and then the complementary primer
14 from the insert. A PCR mix may be used such as the
15 RedTag DNA Polymerase (Sigma Aldrich, Dorset,
16 England) and the thermocycler conditions used may be
17 the standard PCR programme using 50°C as the
18 annealing temperature or adjusted as required.

19
20 Although all colony selecting and picking can be
21 done manually, automated colony pickers are
22 preferred. Automated colony pickers such as the
23 BioRobotics BioPick allow for the uniform and
24 reproducible selection of clones from transformation
25 plates. Clone selection determinants can be set to
26 ensure picking colonies of a standardised size and
27 shape. After picking and plate inoculation,
28 propagation of clones can be carried out as
29 described above.

30
31 Identification of positive clones can be achieved
32 through a variety of methods, including standard

1 techniques such as digestion analysis of plasmid
2 DNA; colony PCR and DNA sequencing Alternatively, in
3 a preferred embodiment, the novel method of dot-
4 blotting described herein for the identification of
5 positive clones may be used in place of such
6 traditional techniques, prior to final confirmation
7 by DNA sequencing. The use of this method in the
8 platform presented here is not essential in the use
9 of this platform over existing screening
10 methodologies, but represents a rapid, reproducible
11 and robust detection method. The protocol described
12 here is a new protocol for an existing method for
13 which commercially available equipment (Bio-Rad
14 DotBlot) can be purchased.

15

16 This particular method is useful for the rapid
17 detection or presence of recombinant protein and
18 allows for a determination of all clones
19 irrespective of solubility and conformation. This
20 is useful at this stage, because conformational
21 structures can inhibit the detection of tag domains
22 if they are not presented properly on the surface of
23 the protein. This can occur as easily with both
24 soluble and insoluble protein.

25

26 For example, after growth on the micro-titre plates
27 is complete, the plate is centrifuged at 4000 rpm
28 for 10 minutes at 4°C to harvest the bacterial
29 cells. The supernatant is removed and the cell
30 pellets are re-suspended in 50 µl lysis buffer (10
31 mM Tris.HCl, pH 9.0, 1mM EDTA, 6 mM MgCl₂)
32 containing benzonase (1 µl/ml). The plate is

1 subsequently incubated at 4°C with shaking for 30
2 minutes. A sample (10 µl) of the cell lysate is
3 added to 100 µl buffer (8 M urea, 500 mM NaCl, 20 mM
4 sodium phosphate, pH 8.0) and incubated at room
5 temperature for 20 minutes. Samples are then
6 applied to a BioDot apparatus (BioRad) containing
7 nitrocellulose membrane (0.45µM pore size) in
8 accordance with the manufacturers' instructions.
9 The membrane is removed and transferred into
10 blocking reagent (3% w/v; Bovine serum albumin in
11 TBS) for 30 minutes at room temperature. The blot
12 is washed briefly with TBS then incubated in a
13 primary antibody, specific to the tag being used for
14 the subset of expression clones. Depending on the
15 nature of the primary i.e., whether or not it has a
16 horse radish peroxidase (HRP) reporter function,
17 will depend on whether the use of a secondary is
18 required. For detection of specific binding the
19 membrane is then washed 2x 5 minutes in TBS followed
20 by 1x 5 minute wash in 10 mM Tris.HCl pH7.6.
21 Detection of specifically bound antibody is
22 disclosed by the addition of chromogenic substrate
23 (6 mg diaminobenzidine in 10 ml 10 mM Tris.HCl pH
24 7.6 containing 50 µl 6% H₂O₂) . The reaction is
25 stopped by thorough rinsing in water. Positive
26 clones identified by this procedure can then be
27 confirmed by DNA sequencing of the expression
28 construct using now industry-standard techniques and
29 equipment such as ABI and Amersham Biosciences.

30

31 Sequencing

32

1 The sequencing reactions may be performed using
2 techniques common in the art using any suitable
3 apparatus. For example, sequencing may be performed
4 on the cloned inserts, using the Big Dye Terminator
5 cycle sequencing kits (Applied Biosystems,
6 Warrington, UK) and the specific sequencing primer
7 run on a Peltier Thermal cycler model PTC225 (MJ
8 Research Cambridge, Mass). The reactions may be run
9 on Applied Biosystems - Hitachi 3310 Sequencer
10 according to the manufacturer's instructions. These
11 sequences are checked to ensure that no PCR
12 generated errors have occurred.

13

14 Assessment of Solubility of Positive Clones

15

16 The cells of positive clones may be harvested and
17 soluble and insoluble protein detected.

18

19 Any suitable techniques known in the art can be used
20 to separate soluble and insoluble protein, such as
21 the use of centrifugation, magnetic bead
22 technologies and vacuum manifold filtrations.

23 Typically, however, the separated proteins are
24 ultimately analysed by acrylamide gel and western
25 blotting. This confirms the presence of recombinant
26 protein at the correct size.

27

28 In one embodiment, contents of each well in the 96
29 well plate are transferred into a Millipore 0.65 μ m
30 multi-screen plate. The plate is placed on a vacuum
31 manifold and a vacuum is applied. This draws off
32 the culture medium to waste. The cells are then

1 washed with PBS (optional), again the vacuum is
2 applied to remove the PBS. The multi-screen plate is
3 removed from the manifold and bacterial cell lysis
4 buffer (containing DNase) (50 μ l) is added to each
5 well. The plate is incubated at room temperature
6 for 30 minutes with shaking to facilitate lysis of
7 the cells. A fresh 96 well microtitre plate (ELISA
8 grade) is placed inside the vacuum manifold and the
9 multi-screen plate is placed above it. When a
10 vacuum is applied the contents of each well are
11 drawn into the micro-titre plate below. The vacuum
12 only needs to be applied for 20 seconds. The
13 collected lysate contains the soluble fraction of
14 expressed protein. A sample of the collected lysate
15 may subsequently analysed by SDS-PAGE and Western
16 blotting to confirm both the presence and correct
17 molecular weight of the target protein.

18
19 The use of SDS-PAGE and Western blotting can be
20 expensive and time consuming, especially when
21 numerous samples must be analysed for each
22 construct. In light of this we have developed a
23 protocol whereby one gel can be used for both total
24 protein staining and western blotting. This
25 represents a significant improvement in this
26 methodology and obviously allows cost saving, and
27 precise comparisons can be made with regard to total
28 protein and western blotting as both sets of results
29 come from the one gel.

30

31 The basis of this protocol is in the ability to use
32 chloroform and UV light to stain protein on an SDS-

1 PAGE gel (Kazmin et al., Anal Biochem, 2001, 301(1)
2 91-6; doi:10.1006/abio.2001.5488). We have used
3 this technique to great effect as it allows for the
4 extremely rapid staining of a SDS-PAGE gel in less
5 than a tenth of the time taken using other more
6 traditional staining methods such as Commassie
7 Brilliant Blue and Collodial Blue stains. We then
8 decided to take this observation a step further and
9 analyse the ability of a chloroform-stained gel to
10 be used in Western blotting. This would not be
11 expected to work as other stained gels result in the
12 fixing of the protein to the gel and subsequent
13 inability to transfer the protein during blotting.
14 This expectation is coupled to the fact that
15 chloroform is not compatible with western blotting
16 equipment (Bio-Rad SD blotter user's manual).
17 However, fortuitously, we have discovered that with
18 a wash of the chloroform-stained gel in double-
19 distilled water, to remove excess chloroform, and
20 after subsequent soaking in transfer buffer,
21 proteins were effectively transferred during western
22 blotting in contrast to expectations. This transfer
23 was no-less effective than from a gel that has not
24 been pre-stained with chloroform and UV light.
25 Figure 6 primarily shows results relating to the
26 production of soluble protein by the platform, but
27 also shows the ability to use the chloroform-stained
28 SDS-PAGE derived western blot for the identification
29 of proteins, without any apparent damage caused to
30 the proteins.
31

1 The use of a chloroform-stained SDS-PAGE derived
2 western blot for the identification of proteins
3 forms another aspect of the present invention.

4

5 Scale-Up and Purification

6

7 This analysis provides a picture of the expression
8 status of the clones on each plate. Using this
9 analysis, positive soluble protein expressing clones
10 can be identified for the production of soluble
11 recombinant protein for a given target protein. The
12 clones may be selected and their growth scaled up
13 e.g. to 5 ml scale, using the saved primary plate as
14 an inoculum. Parameters that may be taken into
15 consideration in deciding on the appropriate culture
16 to select for scale-up include the desirability of
17 specific regions for the production of an antigen,
18 the overall expression levels of the clone and
19 factors that may affect affinity purification such
20 as amino acid composition.

21

22 Example 1. Expression construct design

23

24 Figure 1 is a diagrammatic representation of the
25 protein Jak1. Using pfam, the position of distinct
26 domains was established. Further analysis of these
27 domains was then carried out using Tmpred and the
28 Kyle and Dolittle hydrophobicity algorithm to
29 determine the usefulness of these domains as soluble
30 antigens. From this tentative analysis, four
31 domains were selected for amplification and
32 expression analysis. Based on this preliminary in,

1 *silico* analysis, primers specific for a target
2 protein were designed and used to amplify domains
3 selected for analysis.

4

5 Vectors (500 ng) were restricted with *Bam*HI (20
6 units) and *Sal*I (20 units) in the presence of calf
7 intestinal alkaline phosphatase (CIP) (2 units), gel
8 purified and quantified using standard methods.

9 Purified PCR fragments (100 ng) were restricted with
10 *Bam*HI (5 units) and *Sal*I 5 units), gel purified,
11 quantified, and then used in a ligation reaction
12 with the restricted vector again using standard T4
13 DNA ligase methods (Ready-to-Go T4 DNA ligase,
14 Amersham Biosciences). A sample of the ligation
15 reaction (1 µl) was then used to transform the
16 appropriate competent bacterial cells (TOP10F' were
17 used here for the pQE based vectors, a modification
18 of the manufacturers recommendations; BL21(DE3)pLySE
19 for pET43.1a and TOP10F' for pGEX-Fus).
20 Transformants were selected on LB/ampicillin (100
21 µg/ml) overnight at 28°C.

22

23 A Cambridge BioRobotics BioPick instrument was used
24 for the picking of 24 colonies from each of the
25 transformant plates into flat-bottomed and lidded
26 micro-titre plates. The clones were used to
27 inoculate 150 µl of LB (containing 100µg/ml
28 ampicillin), and these were allowed to grow
29 overnight at 37 °C.

30

31 A secondary plate was prepared by the inoculation of
32 200 µl of LB containing the required supplements

1 with 10 µl of the overnight primary culture. These
2 were then grown at 37 °C. Once an optical density
3 (OD) of 0.25 at A550 was reached, IPTG (final
4 concentration, 1 mM) was added to induce expression
5 of the recombinant protein. Culture propagation was
6 continued for another 4 hours prior to harvesting of
7 bacterial cells.

8
9 After clones expressing specific recombinant protein
10 have been identified, the solubility of these
11 proteins has to be established prior to clone
12 selection for purification. This can be performed a
13 number of ways including the use of centrifugation
14 and automation-friendly vacuum manifold separations.
15 The results here were obtained using methodologies
16 based around the use of vacuum-assisted filtration
17 to separate soluble and insoluble protein. The
18 filtrates that were produced from the method
19 described were then analysed by SDS-PAGE and Western
20 blotting to confirm the production of a recombinant
21 protein of the correct anticipated molecular weight.

22 23 Example 2 Design and Construction of SNUT Expression 24 Tag

25
26 Based on analysis of the amino acid sequence and
27 predicted structure of SrtA_{ΔN}, it was decided to
28 amplify the region of amino acids 26 to 171 of the
29 SrtA sequence. Amplification was conducted using
30 the forward primer 5' TTTTGTAGATCTAAACCACATATCGAT
31 and the reverse primer 5'
32 TTTTGTGGATCCATCTAGAACTTCTAC. This product was then

1 digested with *Bgl*I and *Bam*HI and ligated into pQE30
2 vector which had also been digested with *Bam*HI to
3 form the pSNUT vector. The ligation mix was
4 transformed into TOP10F' cells and single colonies
5 propagated on LB agar containing 100 µg/ml
6 ampicillin. Clones with the *srtA* fragment in the
7 correct orientation were screened by expression
8 analysis and positive clones identified using the
9 denaturing dot-blot assay described earlier.

10

11 The sequence encoding the SNUT tag was cloned into
12 pQE30 as described earlier and positive clones
13 identified by denaturing dot blots, SDS-PAGE and
14 Western blotting. Final confirmation of these
15 clones was provided by DNA sequencing, and the
16 sequence of the multiple cloning region of the
17 resultant vector is shown in Figure 4. Variances in
18 the sequence of the SNUT domain were observed from
19 the sequence for *SrtA* that has been logged in
20 Genbank (AF162687). The variances are (using the
21 annotation of AF162687) nucleotide 604 AAG causing
22 an amino acid mutation of KAR; nucleotide 647 AAG,
23 codon remains K, therefore a silent mutation;
24 nucleotide 966 GAA causing an amino acid mutation of
25 GAQ.

26

27 Example 3 Trials of SNUT Expression Constructs

28

29 Target inserts were cloned into the pSNUT vector
30 using primer construction and digestion of resulting
31 PCR amplifications with *Bam*HI and *Sal*I as described
32 earlier. pSNUT was digested with *Bam*HI in a similar

1 manner and the target inserts cloned as described.
2 Clones were screened using the denaturing dot-blot
3 system and then analysed with SDS-PAGE and western
4 blotting. Positive clones were used for preparative
5 200 ml LB cultures containing 100 µg/ml ampicillin
6 and induced as described earlier. This was grown to
7 an optical density of 0.5 at A₅₅₀ at 37 °C.
8 Expression of SNUT was then induced with the
9 addition of IPTG (final concentration, 1 mM) and
10 left to grow for another 4 hours. Cells were then
11 harvested by centrifugation at 5K rpm for 15
12 minutes. Cells were re-suspended in 30 ml PBS
13 containing 0.1% Igepal and lysis induced by two
14 freeze-thaw cycles. The suspension was then
15 sonicated and centrifuged at 5K rpm for 15 minutes.
16 The soluble supernatant was transferred to a fresh
17 container and filtered through a 0.8 µm disc filter
18 to remove final cell debris. This solution was then
19 applied to a Ni²⁺ charged IMAC column (Amersham
20 Biosciences HiTrap Chelating column, 1 ml) using an
21 AKTA Prime low pressure chromatography system and
22 column was then treated using a standard native his-
23 tag purification protocol involving washing of
24 column with 20 mM sodium dihydrogen phosphate pH 8.0
25 containing 10 mM imidazole, 500 mM NaCl, and elution
26 of soluble his-tagged proteins using 20 mM sodium
27 dihydrogen phosphate pH 8.0 containing 500 mM
28 imidazole, 500 mM NaCl. Elution fractions were then
29 analysed on an SDS-PAGE gel (4-20% SDS-PAGE Bio-Rad
30 Criterion gel), which was stained with chloroform as
31 described earlier. This gel was then subsequently
32 western blotted and the his-tagged protein detected

1 with anti-poly-histidine monoclonal antibody using
2 the techniques described herein.

3
4 Preliminary trials and native purification showed
5 that the SNUT fragment was very soluble and its
6 characteristics were in no way diminished by
7 truncation, thus showing that SNUT could represent a
8 useful tag domain (data not shown). To fully test
9 the abilities of SNUT, we then chose two proteins
10 for which soluble protein production had proved
11 impossible using the other expression systems in
12 which SNUT was not used as a tag. These were murine
13 MAR1 and human Jak1. Clones were prepared and
14 selected using the method as described in the
15 Examples above and positive clones were subsequently
16 grown and induced at 37 °C. These were then treated
17 to identical native histag purifications. Both
18 proteins behaved very favourably under standard
19 purification conditions as can be seen from the
20 purification profiles in Figure 5. For both these
21 trial proteins, this was the first example of such
22 purification under soluble conditions. The
23 production of these proteins using conventional
24 techniques has failed to produce any soluble
25 protein, irrespective of expression system or growth
26 conditions used (data not shown). However, as
27 described in this example, when the protein
28 fragments were expressed in pSNUT, soluble proteins
29 can be surprisingly obtained.

30
31 The effectiveness of SNUT as a fusion protein is
32 even more significant when it is considered that no

1 special growth conditions were required for the
2 generation of soluble protein. This is remarkable
3 when one considers the protein expressionist's
4 standard GST tag which is not even soluble itself
5 when expressed at 37 °C; 28 °C is required before
6 even the generation of GST on its own without any
7 target protein is observed.

8

9 All documents referred to in this specification are
10 herein incorporated by reference. Various
11 modifications and variations to the described
12 embodiments of the inventions will be apparent to
13 those skilled in the art without departing from the
14 scope and spirit of the invention. Although the
15 invention has been described in connection with
16 specific preferred embodiments, it should be
17 understood that the invention as claimed should not
18 be unduly limited to such specific embodiments.
19 Indeed, various modifications of the described modes
20 of carrying out the invention which are obvious to
21 those skilled in the art are intended to be covered
22 by the present invention.

23

1 Claims

2

3 1. Use of a sortase gene product as a purification
4 tag.

5

6 2. The use according to claim 1 wherein the
7 sortase gene product is a *Staphylococcus aureus*
8 srtA gene product.

9

10 3. The use according to claim 1 or claim 2 wherein
11 the sortase gene product is encoded by the
12 nucleotide sequence shown in Figure 4 or a
13 variant or fragment thereof.

14

15 4. The use according to any one of claims 1 to 3
16 wherein the sortase gene product comprises
17 amino acids 26 to 171 of the SrtA sequence
18 shown in Figure 4 or a variant or fragment
19 thereof.

20

21 5. An expression construct for the production of
22 recombinant polypeptides, which construct
23 comprises an expression cassette consisting of
24 the following elements that are operably
25 linked: a) a promoter; b) the coding region of
26 a DNA encoding a sortase gene product as a
27 purification tag sequence; and c) a cloning
28 site for receiving the coding region for the
29 recombinant polypeptide to be produced; and d)
30 transcription termination signals.

31

- 1 6. The expression construct according to claim 5
2 wherein the sortase gene product is a
3 *Staphylococcus aureus* srtA gene product.
4
- 5 7. The expression construct according to claim 5
6 or claim 6 wherein the sortase gene product is
7 encoded by the nucleotide sequence shown in
8 Figure 4 or a variant or fragment thereof.
9
- 10 8. The expression construct according to any one
11 of claims 5 to 7 wherein the sortase gene
12 product comprises amino acids 26 to 171 of the
13 SrtA sequence shown in Figure 4 or a variant or
14 fragment thereof.
15
- 16 9. A method for producing a polypeptide,
17 comprising: a) preparing an expression vector
18 for the polypeptide to be produced by cloning
19 the coding sequence for the polypeptide into
20 the cloning site of an expression construct as
21 claimed in any one of claims 5 to 8; b)
22 transforming a suitable host cell with the
23 expression construct thus obtained; and c)
24 culturing the host cell under conditions
25 allowing expression of a fusion polypeptide
26 consisting of the amino acid sequence of the
27 purification tag with the amino acid sequence
28 of the polypeptide to be expressed covalently
29 linked thereto; and d) isolating the fusion
30 polypeptide from the host cell or the culture
31 medium by means of binding the fusion

- 1 polypeptide present therein through the amino
2 acid sequence of the purification tag.
3
- 4 10. The method according to claim 9, wherein the
5 sortase gene product is a *Staphylococcus aureus*
6 *srtA* gene product.
7
- 8 11. The method according to claim 9 or claim 10
9 wherein the sortase gene product is encoded by
10 the nucleotide sequence shown in Figure 4 or a
11 variant or fragment thereof.
12
- 13 12. The method according to any one of claims 9 to
14 11 wherein the sortase gene product comprises
15 amino acids 26 to 171 of the *SrtA* sequence
16 shown in Figure 4 or a variant or fragment
17 thereof.
18
- 19 13. A fusion polypeptide obtained by the method of
20 any one of claims 9 to 12.
21
- 22 14. A purification tag comprising a sortase gene
23 product.
24
- 25 15. The purification tag according to claim 14
26 wherein the gene product is a *Staphylococcus*
27 *aureus srtA* gene product.
28
- 29 16. The purification tag according to claim 14 or
30 claim 15 wherein the sortase gene product is
31 encoded by the nucleotide sequence shown in
32 Figure 4 or a variant or fragment thereof.

1
2 17. The purification tag according to any one of
3 claims 14 to 16 wherein the sortase gene
4 product comprises amino acids 26 to 171 of the
5 SrtA sequence shown in Figure 4 or a variant or
6 fragment thereof.
7
8

Figure 1

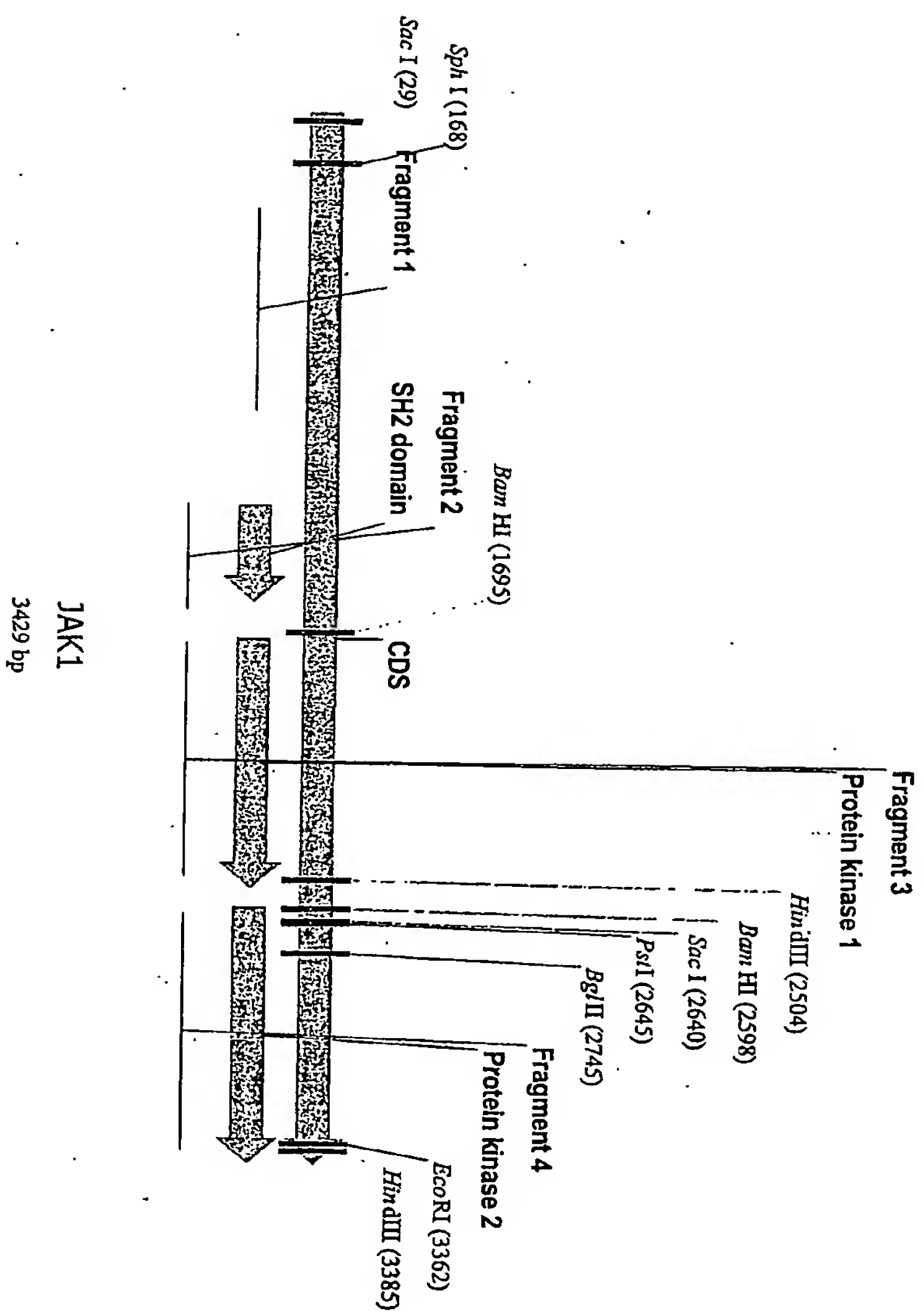


Figure 2

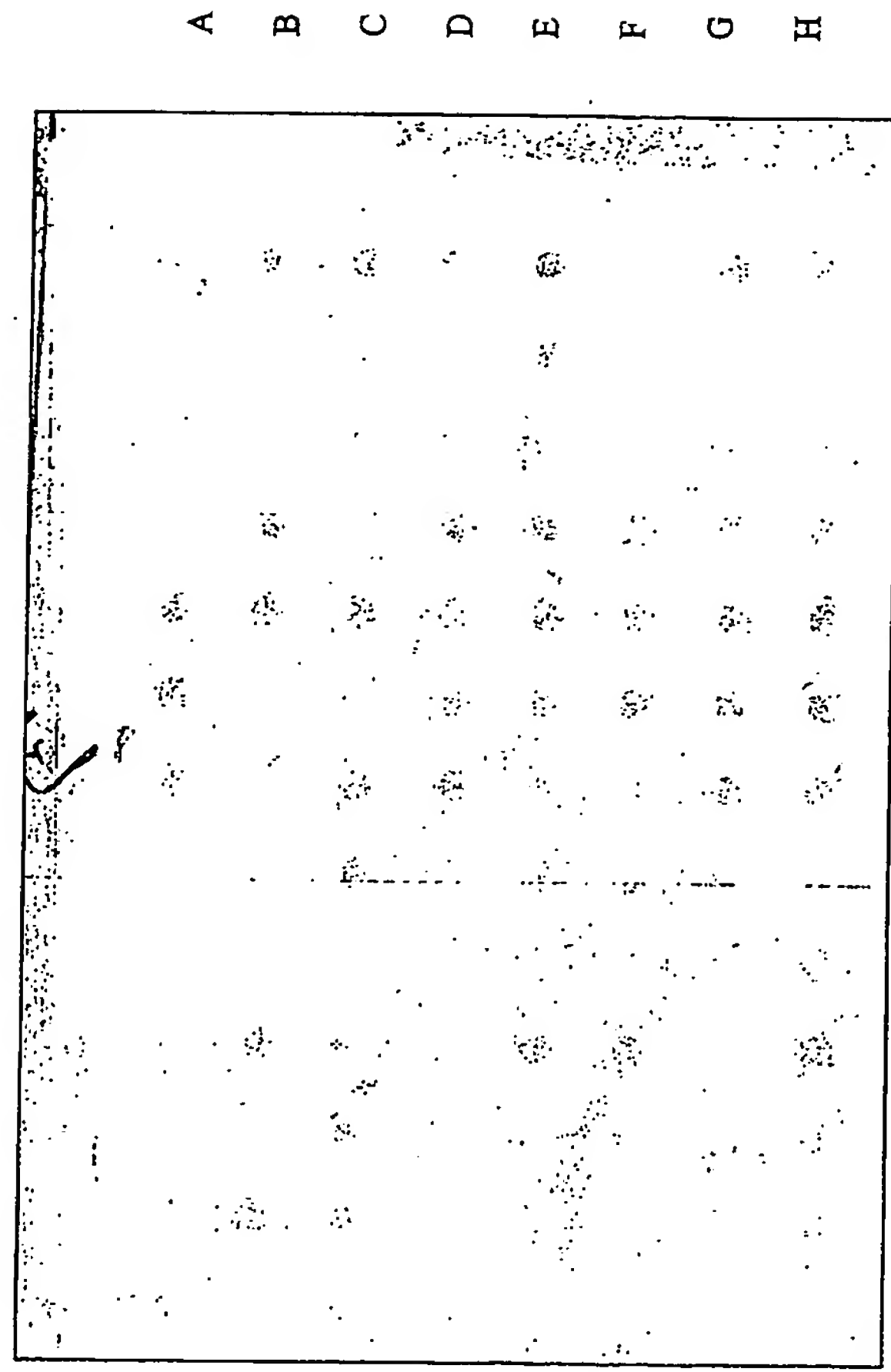


Figure 3

3/6

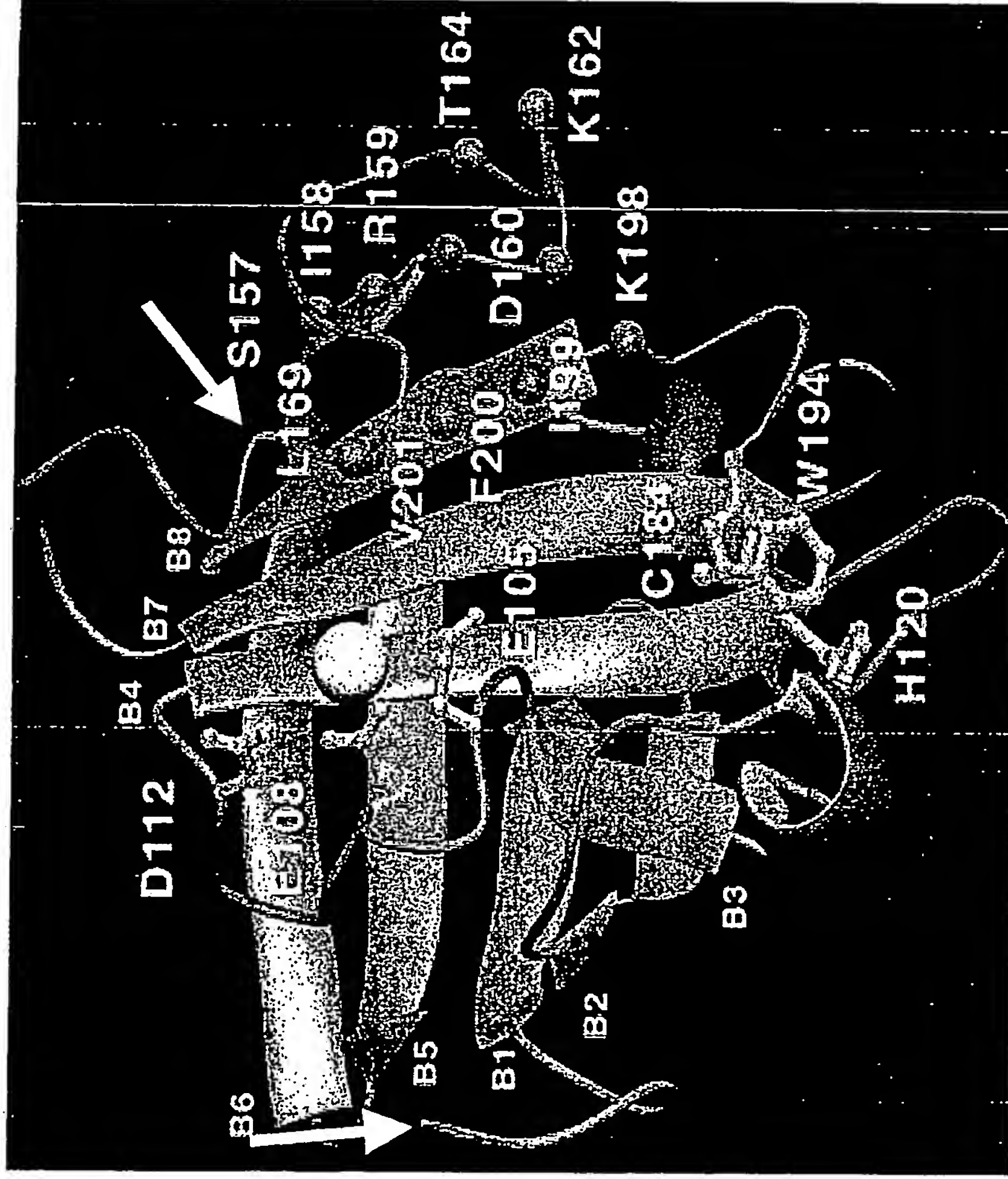


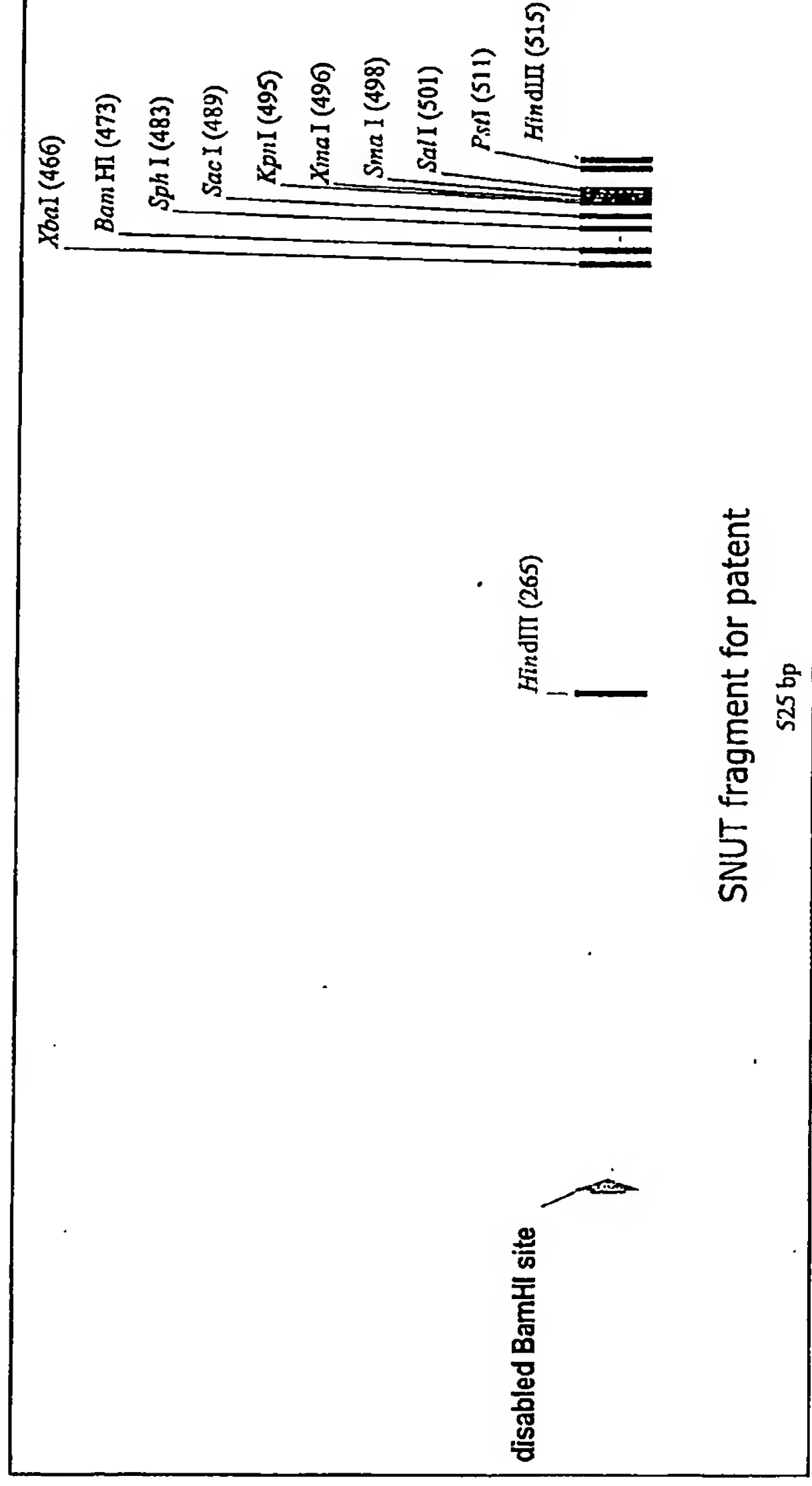
Figure 4a

a

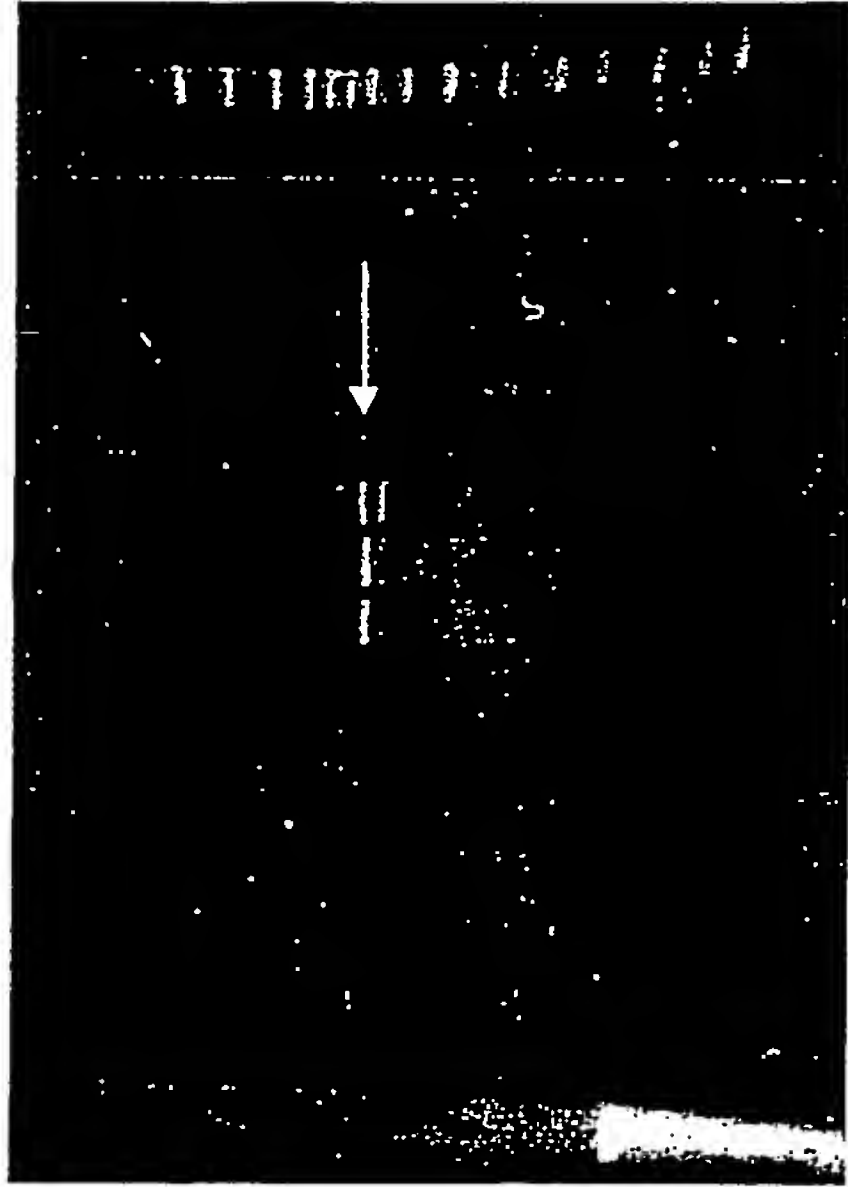
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TACTCTCCTAGCGTAGTGGTAGTGGTAGTGCCTAGATTGGTGTATAGCTATTATAGAA
M R G S H H H H H H H G S K P H I D N Y L
CAGGATAAGATAAAGATGAAAGGATTGAACAATATGATAAAATGTAAAGACAGGCG 120
GTGCTATTCTATTCTACTTTCCTAACTTGTATATACTATTTTACATTTCTTGTCCGC
H D K D K D E R I E Q Y D K N V K E Q A
AGTAAGGATAAAAAGCAGCAAGCTAAACCTCAAAATTCGAAAGATAAAATCGAAAGTGCA 180
TCATTCCCTATTTTTCGTCGTTTCGATTGGAGTTTAAGGCTTTCTATTTAGCTTTCACCGT
S K D K K Q Q A K P Q I P K D K S K V A
GGCTATATTGAAATCCAGATGCTGATATTAAGAACCAGTATATCCAGGACCAGCAACA 240
CCGATATAAACTTTAAGGCTCTACGACTATAATTTCTTGGTCATATAGGTCCTGGTCTGT
G Y I E I P D A D I K E P V Y P G P A T
CCTGAACAATTAATAGAGGTGTAAGCTTTGCAGAAAGAAATGAATCACTAGATGATCAA 300
GGACTTGTTAATTTATCTCCACATTCGAAACGTCTCTTTTACTTAGTACTACTAGTT
P E Q L N R G V S F A E E N E S L D D Q
AATATTTCAATTGCAGGACACACTTTCAATTGACCGTCCGAACTATCAATTTACAAATCTT 360
TTATAAAGTTAAGTCCCTGTGTGAAAGTAACTGGCAGGCTTGATAGTTAAATGTTTAGAA
N I S I A G H T F I D R P N Y Q F T N L
AAAGCAGCCAAAAGGTAGTATGGTGTAATTTAAAGTTGGTAATGAAACACGTAAGTAT 420
TTTCGTCGGTTTTTTCCATCATACCATGAAATTTCAACCATTAATTTGTGCATTGATA
K A A K K G S M V Y F K V G N E T R K Y
AAAATGACAAGTATAAGAGATGTTAAGCCCTACAGATGTAGAAGTTCTAGATGGATCCGCA 480
TTTTACTGTTTCAATTTCTCAATTCGGATGTCTACATCTTCAAGATCTACCTAGGCGT
K M T S I R D V K P T D V E V L D G S A
TGGAGCTCGGTACCCGGGTCGACCTGCAGCCAAAGCTTAATTAG 525
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C E L G T P G R P A A K L N *

Figure 4b

b



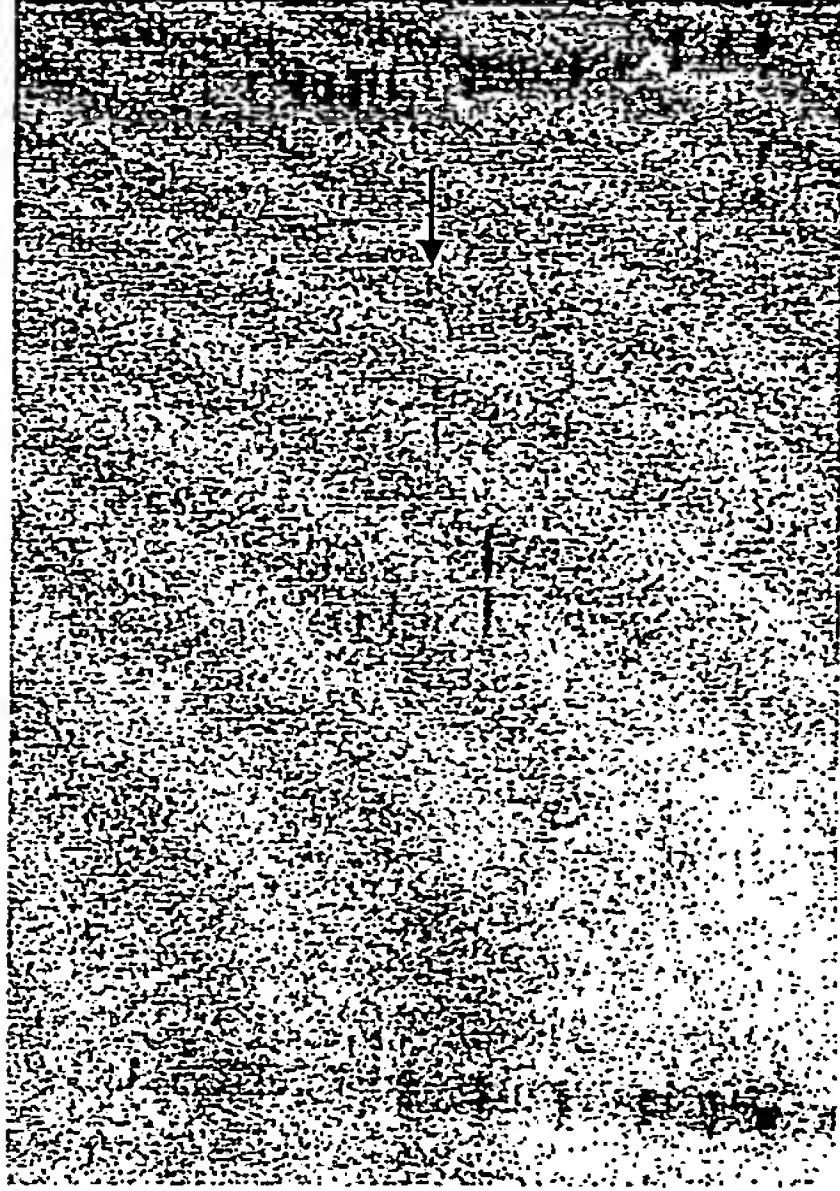
6/6



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

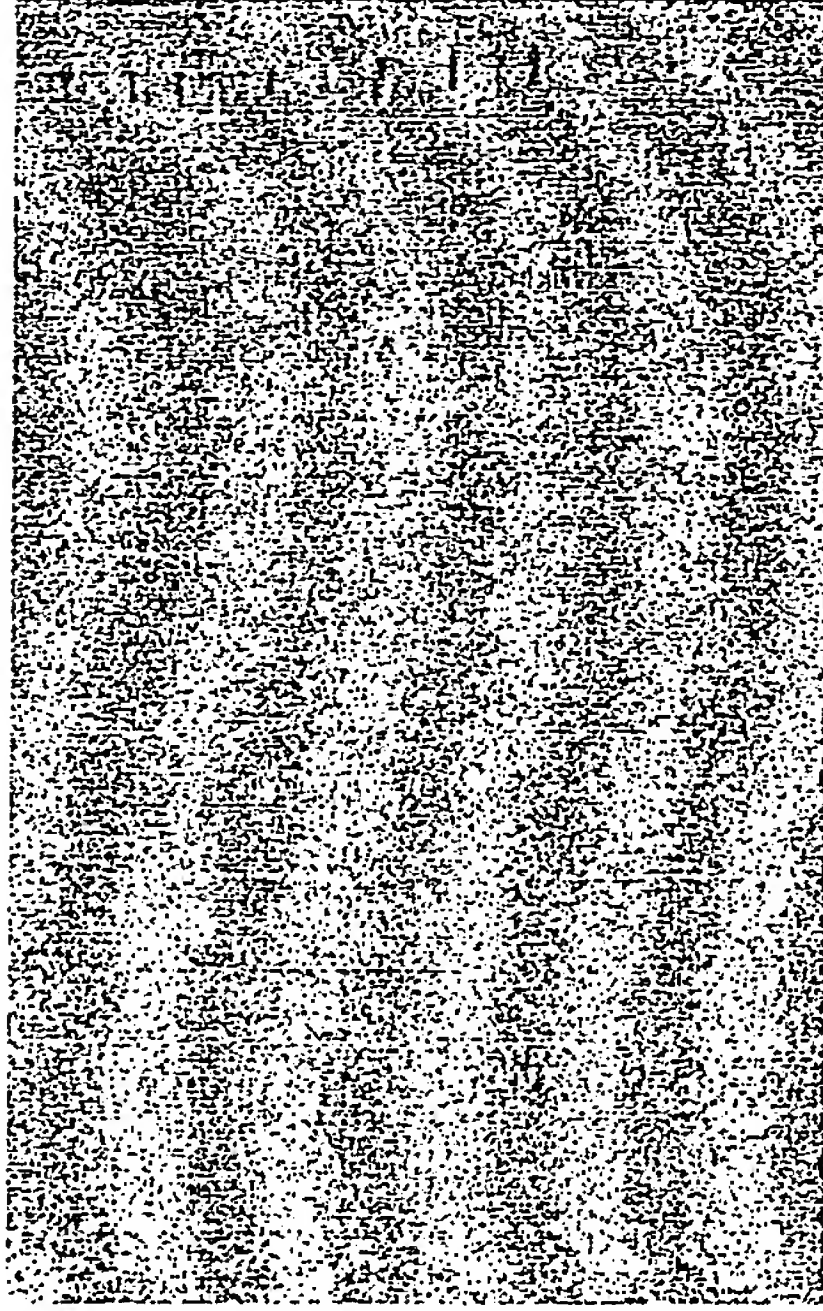
M

b



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 M

c



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 5

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